

2020
PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : G01N 33/545, 33/53	A1	(11) International Publication Number: WO 92/06380 (43) International Publication Date: 16 April 1992 (16.04.92)
(21) International Application Number: PCT/SE91/00649 (22) International Filing Date: 26 September 1991 (26.09.91) (30) Priority data: 9003122-0 1 October 1990 (01.10.90) GB (71) Applicant (for all designated States except US): PHARMACIA BIOSENSOR AB [SE/SE]; S-751 82 Uppsala (SE). (72) Inventors; and (75) Inventors/Applicants (for US only) : LÖFÅS, Stefan [SE/SE]; Svartbäcksgatan 99 A, S-753 35 Uppsala (SE). RÖNNBERG, Inger [SE/SE]; Salagatan 41 B, S-753 26 Uppsala (SE). LAGERSTRÖM, Katarina [SE/SE]; Torckelsgatan 2 B, S-753 26 Uppsala (SE).		(74) Agents: WIDÉN, Björn et al.; Kabi Pharmacia AB, S-751 82 Uppsala (SE). (81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US. Published <i>With international search report.</i>
(54) Title: IMPROVEMENT IN SOLID PHASE BINDING ASSAY (57) Abstract <p>An assay method comprises the step of binding a second substance to a first substance immobilized to a solid phase surface, the first substance being either a ligand or a species bound directly or indirectly thereto. The method is characterised in that the solid phase surface having the first substance immobilized thereto exhibits an electric charge, and that the reaction for binding the second substance to the first substance is performed at an ionic strength below 100 mM, and at such pH conditions that the electric charge of the second substance is opposite to that of the solid phase surface so that the second substance is electrostatically attracted by the solid phase surface to be concentrated thereto.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU ⁺	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE*	Germany	MC	Monaco	US	United States of America
DK	Denmark				

+ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

IMPROVEMENT IN SOLID PHASE BINDING ASSAY

The present invention relates to an improvement in assay methods comprising the specific binding of a second
5 substance to a first substance already bound to a solid phase surface.

In our WO 90/05303 there are disclosed sensing surfaces capable of selective biomolecular interactions and designed to be used in biosensor systems, particularly
10 systems based upon surface plasmon resonance (SPR). In this type of optical biosensors changes in the refractive index in a layer close to a thin metal film are detected by the consequential changes of the intensity of a totally reflected light beam. For a more detailed description of
15 such a biosensor it is referred to our WO 90/05295 relating to an optical biosensor system, and our WO 90/05305 relating to a sensor unit and its use in biosensor systems.

The above mentioned sensing surfaces comprise a film of a free electron metal, preferably silver or gold, having
20 one of its faces coated with a densely packed monolayer of specific organic molecules. To this monolayer a biocompatible porous matrix, e.g. a hydrogel, is bound, which matrix is employed for immobilizing a suitable ligand for a target biomolecule to be determined by the particular
25 biosensor.

To make the matrix bind a desired ligand, it is activated by the introduction of suitable functional groups, the matrix thereby normally obtaining a positive or negative net charge. Provided that the ligand to be bound,
30 typically a protein, is of the opposite charge, the electrostatic interaction between the ligand and the matrix will concentrate the ligand at the surface and thereby provide for efficient binding of the ligand to the surface. Such binding to electrically charged surfaces has been
35 well-known in the art for a long time, such as in enzyme immobilization and affinity chromatography (see e.g. US-A-4,829,009, WO 83/02954, and J. Immunol. Methods 1988, vol. 111(2), p. 157-66).

Although such an electrostatic effect is advantageous for the binding of ligands, the possible presence of a residual charge at the solid surface after the ligand binding has, however, hitherto been believed to be a possible source of problems in the subsequent analytical steps because of undesired ionic interactions with the sample.

It has now, in accordance with the present invention, surprisingly been found that the provision of a residual charge at the solid surface remaining after the binding of a ligand thereto may be favourably utilized to facilitate and optimize the binding of an analytical species in the subsequent steps of a particular assay to be performed at the solid phase surface. In the present context the term "analytical species" means any reactive species used in the assay except the initially bound ligand. Thus, for example, in a sandwich type assay, the so-called secondary antibody (in SPR-assay serving as an "enhancement agent") may be efficiently concentrated to the matrix by the created electrostatic interaction resulting in a substantially complete binding to the primary analyte already bound to the ligand, provided that, on one hand, the matrix has an original charge sufficient to leave a residual charge after the immobilization of the ligand thereto, and, on the other hand, that the reaction conditions are selected properly in terms of ionic strength and pH.

Thus, for the purposes of the present invention the reaction conditions involve low ionic strength and an appropriate pH of the reaction medium to ensure a negative or positive charge of the secondary antibody opposite to the charge of the solid phase surface, in contrast to the physiological conditions used in the prior art, i.e. medium ionic strength and about neutral pH. For instance, in the case of a residual negative charge of the matrix, the pH should be selected below the isoelectric point of the antibody to make the latter positively charged, and vice versa. Under these conditions the electrostatic interactions between such opposite charges of the matrix

and the secondary antibody will be utilized without too many screening ions being present. It is also believed that under such conditions the matrix per se will be more open due to internal repelling in the polymer layer, thereby
5 providing for an increased availability of the bound analyte.

To ensure that only the desired specifically active substance is bound to the surface, the importance of which of course depends on the particular application, it may
10 subsequently to the binding step, promoted by the electrostatically caused concentration of secondary reagent in accordance with the invention, be necessary to subject the surface to moderate or high ionic strength conditions to remove species which have only bound electrostatically.

15 The improvements resulting from performing an assay as above in accordance with the present invention in contrast to normal conditions will reside in (i) higher sensitivity and larger dynamic range of the assay, (ii) considerably reduced consumption of secondary antibody, and (iii) faster
20 analyses.

It will be appreciated that the electrostatic interaction derived concentrating effect described above may be utilized not only in other types of SPR-assays than sandwich assays, as will be further explained below, but
25 also more generally in analytical methods on the whole, such as, e.g., per se conventional ELISA type analyses, as long as they involve the use of a solid phase modified or capable of being modified by a charged matrix of suitable type.

30 In its broadest aspect the present invention thus relates to an assay method comprising the step of binding a second substance to a first substance immobilized to a solid phase surface, the first substance being either a ligand or a species bound directly or indirectly thereto,
35 such as an analyte or a secondary reagent bound to the analyte, the method being characterised in that the solid phase surface which has the first substance immobilized thereto exhibits an electric charge, and that the reaction

for binding the second substance to the first substance is performed at an ionic strength below 100 mM, and at such pH conditions that the electric charge of said second substance is opposite to that of the solid phase surface so
5 that the second substance is electrostatically attracted by the solid phase surface.

As mentioned above the method may include the step of subjecting the surface to higher ionic strength conditions after the binding of the second substance in order to
10 remove unspecific binding.

It is to be noted that although the invention basically is intended to be applicable to all steps in an assay subsequent to the immobilization of the ligand, the second substance, especially in immunological assay
15 contexts, is mostly not the analyte to be determined in the assay, since the sample to be tested does not usually comply with the required conditions as to ionic strength and/or pH and it is usually not desired to disturb the sample environment, e.g. for a serum sample. In certain
20 cases, however, the electrostatic concentrating effect of the invention may be utilized also for the binding of the analyte depending on the particular sample.

Thus, in one embodiment of the present invention the second substance is the secondary component in a sandwich
25 type assay, as already discussed above.

In another embodiment the second substance is an agent used for blocking residual binding sites after the immobilization of the ligand to the surface, which sites would otherwise disturb a particular analytical procedure.

30 In still another embodiment the second substance is an analyte, the method of the invention e.g. being used to determine the amount of activity (ligands) that are immobilized to a surface.

Basically, however, the second substance may be any
35 analytical substance for which the specified reaction conditions may be conveniently applied without any negative practical implications.

The second substance is typically a protein or polypeptide or active fragment thereof, but may generally be any substance susceptible to binding in a desired solid phase assay. As will readily be understood, said second
5 substance is primarily a larger type molecule, e.g. a macromolecule, the diffusion rate being higher for smaller molecules and the effect achieved by the present invention thereby being of less significance.

As is readily appreciated by the person skilled in the
10 art the above mentioned first substance may, especially as an analyte, be for example a protein or polypeptide, such as an antigen or antibody, protein A or G, an enzyme, a lectin, avidin, etc; or a hapten, hormone, sugar, biotin, toxin, vitamin, etc.

15 The above mentioned proteins and polypeptides, and fragments thereof, of course include both natural and synthetic or semisynthetic substances, such as proteins and polypeptides modified by chemical means or by genetic engineering techniques. An example is chimeric, i.e. bi- or
20 polyfunctional molecules, such as bi- or polyfunctional antibodies. For the application of such molecules in biosensor surface contexts it may be referred to our aforementioned WO 90/05305. Also, in e.g. a sandwich type assay the secondary reagent may be a bifunctional antibody
25 or other molecule to permit subsequent reaction with a tertiary reagent.

The solid phase surface layer referred to above will depend on the type of analytical method to be used. For biosensor applications, e.g. SPR or electrochemical
30 biosensors, this layer may be a polymer layer, preferably a hydrogel, such as a dextran layer, bound to a metal surface. For the binding of a ligand thereto, the polymer layer is provided with suitable functional groups, and as a result thereof the surface layer may obtain a residual
35 positive or negative electric charge sufficient for the purposes of the invention, but such electric charge may also be introduced in a separate treatment of the surface layer. Examples of surfaces for biosensor applications are

given in our WO 90/05303 referred to above and the disclosure of which is incorporated by reference herein.

As will appear from the above "low ionic strength" is in the context of the present invention defined as being
5 below 100 mM, the "normal" ionic strength in the typical immunological methods being about 150 mM. Preferably, however, the ionic strength is lower than 50 mM and more preferably below 20 mM. Although the ionic strength in the theoretically ideal situation should be at least near zero,
10 this would not be possible in most practical cases, due e.g. to stability considerations for proteins, etc.

The necessary pH value to ensure a suitable charge of the second substance will, of course, vary with the nature of the particular substance. For example, in the case of a
15 protein the pH to be selected will depend on the isoelectric point (I_p) of the protein, a positive charge being obtained at pH values above I_p and a negative charge being obtained at pH values below I_p . For a particular protein having a specific I_p , the pH to be selected should
20 generally differ from the I_p by at least 0.5 in the desired direction. Some protein samples, such as a polyclonal antibody mixture, may exhibit a pH range rather than a single pH value for its I_p , and in such a case the pH difference given above should, of course, instead relate to
25 the relevant range limit of the pH interval, i.e. at or below 4.5 for a polyclonal mixture having a pH range from 5 to 8. In this context it is also to be noted that the I_p of a specific protein may be modified by per se conventional methods to suit, for instance, a particular charged matrix.

30 The method according to the invention is particularly suitable for immunochemical type assays or analytical methods, especially biosensor related methods. As has already been discussed above, the inventive concept is thus advantageously applicable to the binding of the secondary
35 antibody in an immunological sandwich assay, the sensitivity as well as the dynamic range of the reaction being considerably increased.

Another valuable application of the present invention, also mentioned above, is for efficient blocking of surplus binding sites. This is useful in, for example, so-called epitope mapping by biosensor technology (see e.g. our WO 90/05306 relating to the characterization of macromolecules by means of biosensor technology) where a monoclonal antibody is first bound to a sensing surface having immobilized thereto antibodies capable of generally binding mouse antibodies (e.g. of the type rabbit anti-mouse G1 or F_C). The mapping is then performed by sequentially passing the analyte (antigen) and a second specific monoclonal over the surface, binding of the latter indicating that the two monoclonals bind to different sites on the antigen. As is readily understood the amount of the generally binding antibody immobilized to the surface must be relatively high to permit binding of a sufficient amount of the first monoclonal from the relatively low concentration present in, for example, a culture medium. Since the second monoclonal must not bind to the initially immobilized generally binding antibody, which would be interpreted as a false positive response, the surplus binding sites of the generally binding antibody not occupied by the first monoclonal must be blocked by non-specific antibodies. In accordance with the present invention this may be done in a more efficient and reagent saving way if the above described electrostatic concentrating effect is utilized by providing the blocking antibody in a buffer of low ionic strength and at a pH conferring a charge to the blocking antibody that is opposite to that of the sensing surface matrix.

The same concept may be used to block surplus binding sites at a surface having protein A or G immobilized thereto to make it capable of generally binding polyclonal antibodies. In this case the electrostatic effect may, depending on the pH, be used to improve both the binding of the ligand to the protein A or G, and the subsequent blocking of residual binding sites.

Hereinafter the invention will be described by way of some specific examples, which are given only for the purpose of illustration, and which are not to be construed as limiting in any sense whatsoever. In connection herewith
5 reference will be made to the accompanying drawings, in which:

Fig. 1 is a graph showing the surface change response when binding secondary antibody to a biosensor surface in a sandwich assay for β_2 -microglobulin at high (\blacklozenge) and low
10 (\square) ionic strength, respectively;

Fig. 2 is a similar graph as in Fig. 1 showing the surface change response when binding secondary antibody to a biosensor surface in a sandwich assay for luteinizing hormone (LH) at (i) a secondary antibody concentration of 1
15 mg/ml at high (\square) and low (\blacklozenge) ionic strength; (ii) a secondary antibody concentration of 250 μ g/ml at low ionic strength (\blacksquare); and (iii) a secondary antibody concentration of 50 μ g/ml (\blacklozenge) at low ionic strength;

Figs. 3 and 4 are staple diagrams showing the pH
20 dependency of the surface change response for the binding of secondary antibody to a biosensor surface in a sandwich assay for LH at secondary antibody concentrations of 1.0 mg/ml (Fig. 3) and 50 μ g/ml (Fig. 4), respectively;

Figs. 5 to 7 are staple diagrams showing the surface
25 change response of a biosensor surface for the sequential binding of monoclonal (\blacksquare); blocking antibody (\boxtimes), LH (\boxplus), and the possible subsequent binding of the original monoclonal (\square) for three different monoclonals in culture medium at various concentrations of blocking antibody and
30 at high and low ionic strength, respectively;

Fig. 8 is a similar staple diagram as in Figs. 5 to 7 but with purified monoclonal and with higher concentration of monoclonal added in the second addition than in the first addition thereof;

35 Fig. 9 is a similar staple diagram as in Fig. 8 with purified monoclonal but with the same concentration thereof used for both the first and the second addition of the monoclonal; and

Fig. 10 is a similar staple diagram as in Figs. 5 to 9 for the response at low ionic strength for four different blocking antibody concentrations.

All the assays in the examples were performed with a surface plasmon resonance biosensor system of a type disclosed in our WO 90/05295 (the disclosure of which is incorporated by reference herein) and now commercialized under the trade name BIAcore by Pharmacia Biosensor AB, Uppsala, Sweden. This biosensor system comprises a replaceable sensor unit, a block unit for liquid handling having a conduit system for transporting the reagent and the sample solutions over the sensing surface of the sensor unit, an optical unit which couples incident light rays to the sensing surface and detects the reflected radiation, and an evaluation unit which after calibration transforms the detector signal into a parameter proportional to the amount of substance at the sensing surface. When performing a measurement, a defined sample liquid volume is introduced by injection into a defined conduit section, which liquid volume is then by means of eluent liquid, or drive buffer, forced to pass the sensing surface for optical analysis.

The sensor unit with the sensing surface was a Sensor Chip CM 5 (Pharmacia Biosensor AB, Uppsala, Sweden) which is a glass substrate supported gold film to which is bound a matrix constructed from a composite of a metal-protection layer (an adsorbed monolayer of long-chain 1, ω -hydroxyalkyl thiols) and a covalently bound flexible carboxymethyl-modified dextran hydrogel having a negative charge. For the preparation of such a sensing surface it is referred to our above mentioned WO 90/05303.

The measurements were performed substantially as described in our aforementioned WO 90/05305, the disclosure of which is incorporated by reference herein. The drive buffer used throughout the examples was HBS (Hepes buffer saline), pH 7.4, as specified below. The surface change response in the measurements is expressed in "resonance units" (RU); a response of 1000 RU corresponds to a 0.1° shift in the resonance angle (the angle of the incident

light beam, relative to the sensor surface, at which surface plasmon resonance occurs) or a change of 1 ng/mm² in the surface concentration.

EXAMPLE 1

5 Sandwich assay for β_2 -microglobulin

A sandwich assay for β_2 -microglobulin was performed by first immobilizing to the sensing surface a polyclonal antibody specifically directed against β_2 -microglobulin. This was effected by activating the surface with 0.2 M N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.05
10 M N-hydroxysuccinimide (NHS) in distilled water, applying the antibody in coupling buffer (10 mM sodium acetate, pH 5.0) to the surface, and deactivating any excess of EDC and NHS with 1 M ethanolamine, pH 8.5. In a test cycle the
15 sensing surface was then sequentially passed by a sample containing β_2 -microglobulin (analyte) and a secondary reagent in the form of a polyclonal Ig-fraction (2.5 mg/ml; 10% specific activity), respectively, and the response for the secondary reagent was measured. Each test cycle was
20 terminated by regenerating the surface with 10 mM HCl, pH 2.2. The assay was run with different concentrations of the analyte and at low ionic strength (10 mM Hepes buffer, 0.005% Tween[®] 20, pH 7.5) and high ionic strength (Hepes buffer including 0.15 M NaCl, pH 7.5), respectively, of the
25 secondary reagent. The results are shown in Table 1 below and in Fig. 1 in the drawings.

TABLE 1

5	<u>β_2-globulin</u>	<u>Response of secondary</u>	
	ng/ml	<u>reagent</u>	
		<u>Low i.s.*</u>	<u>High i.s.*</u>
	0	72	173
	100	563	173
	200	1187	417
	400	2190	900
10	800	3360	1888
	1000	3738	1672
	1600	-	1862
	2000	4847	1912

* i.s. = ionic strength

15 As appears from the table and Fig. 1 a more than 3 times higher response for the low ionic strength is obtained for an analyte concentration of 100 ng/ml. At an analyte concentration of about 800 ng/ml the secondary response levels out at high ionic strength, whereas it
20 still increases at 2000 ng/ml at low ionic strength. Since the runs were performed at a pH of 7.5 and the I_p of the secondary antibody is about 8.5, the latter was positively charged at this pH.

EXAMPLE 225 Sandwich assay for luteinizing hormone

A sandwich assay for luteinizing hormone is performed in the same manner as in Example 1 by first immobilizing to the sensing surface a monoclonal antibody specifically directed against luteinizing hormone (LH), and then
30 sequentially passing the LH containing sample (analyte) and a monoclonal anti-LH antibody as the secondary reagent, respectively, over the surface. Samples with different concentrations of the LH-analyte were analyzed by observing the response of the secondary reagent at different
35 concentrations and high and low ionic strength, respectively (Hepes buffer with and without 0.15 M NaCl).

The results are shown in the following Table 2 and in Fig. 2 of the drawings.

TABLE 2

5	<u>Analyte</u> <u>conc.</u> mU/ml	<u>Response of secondary reagent *</u>			
		1 mg/ml	1 mg/ml	250 µg/ml	50 µg/ml
		High i.s.	Low i.s.	Low i.s.	Low i.s.
	0	-24	-17	-6	-7
	0.5	-41	-11	-3	-7
10	2	-36	-1	3	1
	10	-10	49	56	52
	25	26	121	91	*
	100	213	422	447	411
	200	334	570	578	543

15 * Negative values are caused by a slight decrease of the base line level in the course of the assay.

As appears from the table the concentration of the secondary reagent may be reduced by 20 times when buffer of low ionic strength is used in comparison with the corresponding procedure at high ionic strength. Yet both higher sensitivity and greater dynamic is obtained. The higher sensitivity is, for example, observed at the LH-analyte concentration 10 mU/ml when the secondary response is 50 RU for low ionic strength and a secondary reagent concentration of 50 µg/ml, while the same analyte concentration does not give any significant response at high ionic strength and a secondary reagent concentration of 1 mg/ml.

30 Corresponding assay runs were performed to study the pH-dependency of the secondary response, and the results are shown in Figs. 3 and 4; LH was used at a concentration of 1000 ng/ml and two different concentrations of secondary antibody were used, viz. 1.0 mg/ml (Fig. 3) and 50 µg/ml (Fig. 4). Similarly as in Fig. 2, at pH-values above that of the secondary antibody I_p (5.3) the low ionic strength

gives lower responses than those at the high ionic strength. This is due to the antibody then being repelled by the negative charge of the matrix, which effect to a certain extent may be compensated for by higher concentrations. At pH-values below the I_p of the antibody the results are, however, the opposite, the low ionic strength giving a more than doubled response at an antibody concentration of 50 $\mu\text{g/ml}$.

EXAMPLE 3

10 **Blocking of surplus binding sites**

Rabbit anti-mouse G1 antibody (RAMG1) (Pharmacia Diagnostics AB, Uppsala, Sweden), dissolved in 10 mM sodium acetate, pH 5.0, to 30 $\mu\text{g/ml}$, was immobilized to the sensing surface in the same way as in Examples 1 and 2 above. Luteinizing hormone (LH) specific monoclonal, dissolved in HBS (Hepes buffer saline: 10 mM Hepes, 0.15 M NaCl, 3.4 mM EDTA, 0.05% Tween®) was then applied to the surface, whereupon a blocking monoclonal antibody against alphafetoprotein (AFP), dissolved in Hepes-Buffer (10 mM Hepes, 3.4 mM EDTA, 0.05% Tween®) with and without, respectively, NaCl (0.15 M) was passed over the surface to block any residual binding sites not occupied by the LH-monoclonal. LH-analyte (International Enzymes Inc., U.S.A.) dissolved in Pharmacia Diluent (Pharmacia Diagnostics AB, Uppsala, Sweden) to 10 $\mu\text{g/ml}$ was then allowed to react with the surface. To test for the presence of residual non-specific RAMG1 sites, the originally bound monoclonal was again passed over the surface, binding of the monoclonal indicating inefficient blocking.

30 Three different monoclonals, designated 2, 5 and 10, were used. Whereas monoclonals 2 and 5 were used in culture medium, monoclonal 10 was studied both in culture medium and purified at two different concentrations, 7 and 70 $\mu\text{g/ml}$, in HBS-buffer.

35 The blocking antibody was used at high ionic strength (with NaCl) at two concentrations, 1000 and 250 $\mu\text{g/ml}$, and at low ionic strength (without NaCl) at four concentrations, 0, 50, 10 and 250 $\mu\text{g/ml}$.

The results are shown in Figs. 5-10.

As appears from the Figures, the blocking can be effected much more efficiently and reagent saving if the electrostatic concentrating effect of the invention is
5 utilized by providing the blocking antibody in a buffer of low ionic strength and at a pH below the I_p of the antibody (i.e. where it is positively charged). This may, for example, be seen from a comparison of staples 2 and 3 in
Figs. 5-9, wherein 250 $\mu\text{g/ml}$ of non-specific blocking
10 antibody at high and low, respectively, ionic strength are compared.

Fig. 8 illustrates the responses when using purified monoclonal 10 at 7 $\mu\text{g/ml}$ as the first monoclonal and at 70
 $\mu\text{g/ml}$ as the second monoclonal, whereas Fig. 9 shows the
15 responses when the concentration of purified monoclonal 10 is 70 $\mu\text{g/ml}$ in both cases.

In Fig. 10 it is shown that as low blocking antibody concentrations as 25 $\mu\text{g/ml}$ function efficiently at low ionic strength; at 10 $\mu\text{g/ml}$ a small response was obtained
20 when injecting the second monoclonal.

The present invention is, of course, not restricted to the above specifically described embodiments, but many variations and modifications are within the scope of the general inventive concept as stated in the following
25 claims.

CLAIMS

1. An assay method comprising the step of binding a second substance to a first substance immobilized to a solid phase surface, said first substance being either a ligand or a species bound directly or indirectly thereto, characterized in that said solid phase surface having said first substance immobilized thereto exhibits an electric charge, and that the reaction for binding said second substance to said first substance is performed at an ionic strength below 100 mM, and at such pH conditions that the electric charge of said second substance is opposite to that of the solid phase surface so that the second substance is electrostatically attracted by the solid phase surface to be concentrated thereto.
2. A method according to claim 1, characterized in that the step of binding said second substance is followed by a step of subjecting the surface to moderate or high ionic strength conditions.
3. A method according to claim 1 or 2, characterized in that said second substance is selected from possibly modified proteins and polypeptides and fragments and derivatives thereof.
4. A method according to claim 3, characterized in that said second substance is an antibody or a fragment or functional equivalent thereof.
5. A method according to any one of claims 1 to 4, characterized in that the reaction for binding said second substance to said first substance is an immunochemical reaction.
6. A method according to any one of claims 1 to 5, characterized in that said ionic strength is below 20 mM.

7. A method according to any one of claims 1 to 6,
characterized in that said second substance is a secondary
reagent in a sandwich type assay for binding to an analyte
which has first bound to a ligand immobilized to the
5 surface.

8. A method according to any one of claims 1 to 7,
characterized in that said second substance is a blocking
reagent for blocking residual binding sites on a solid
10 phase surface layer after immobilizing a ligand thereto.

9. A method according to any one of claims 1 to 8,
characterized in that said solid phase surface comprises an
electrically charged hydrogel surface layer.

15

10. A method according to any one of claims 1 to 9,
characterized in that said solid phase surface is a sensing
surface for surface plasmon resonance based measurements.

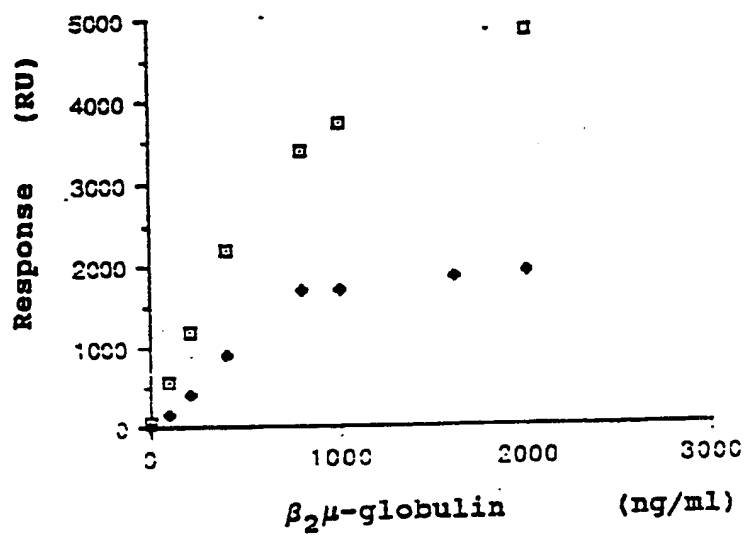


FIG.1

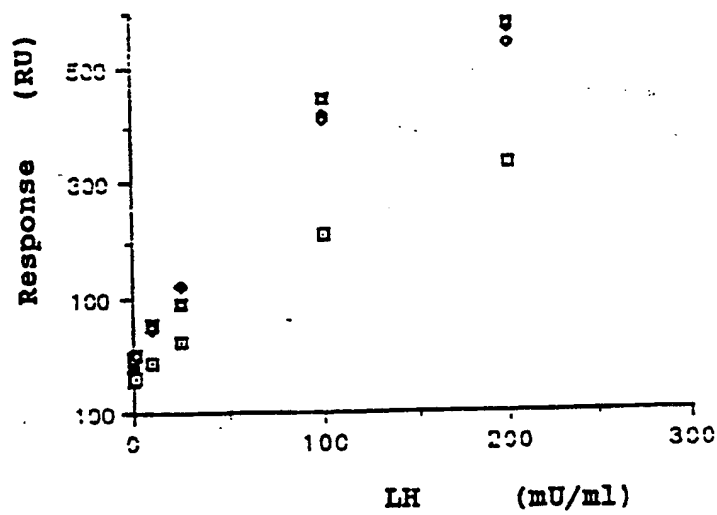


FIG.2

2/5

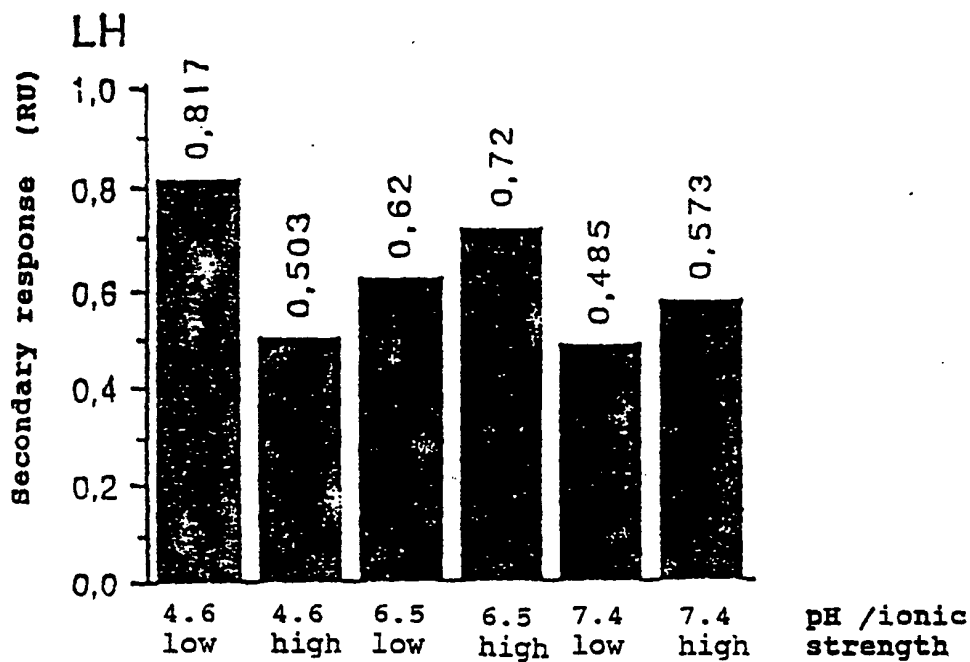


FIG. 3

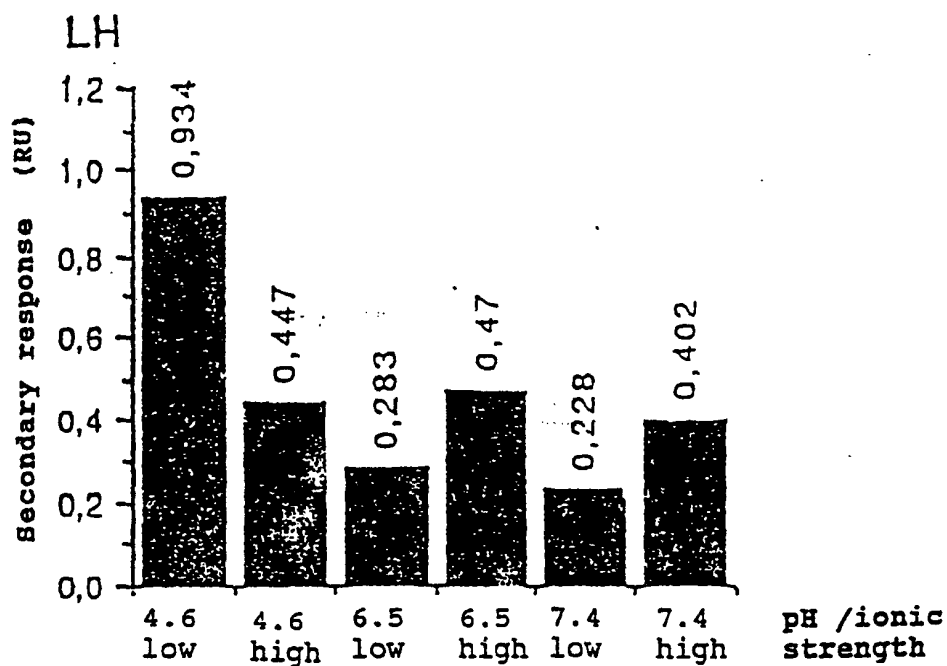


FIG. 4

3/5

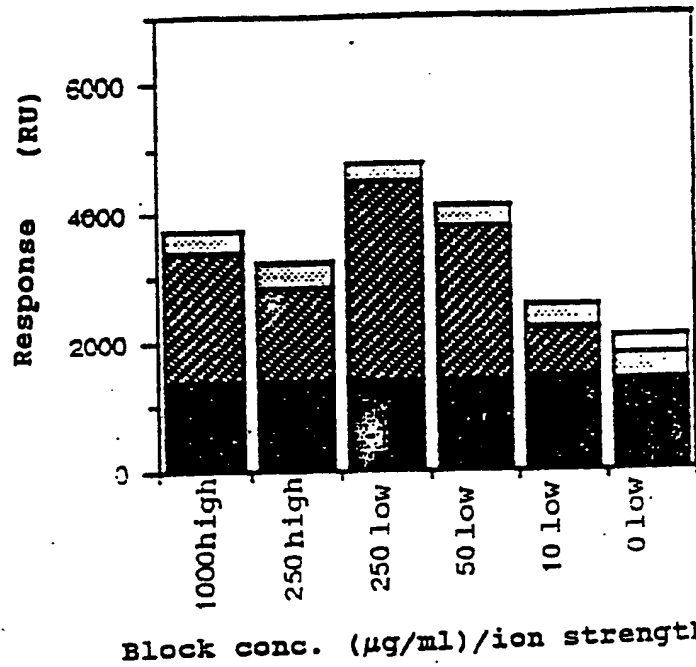


FIG. 5

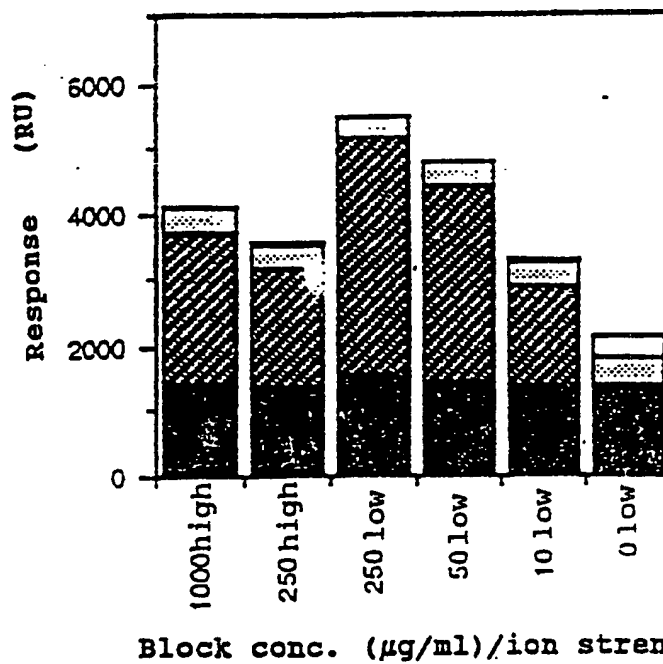
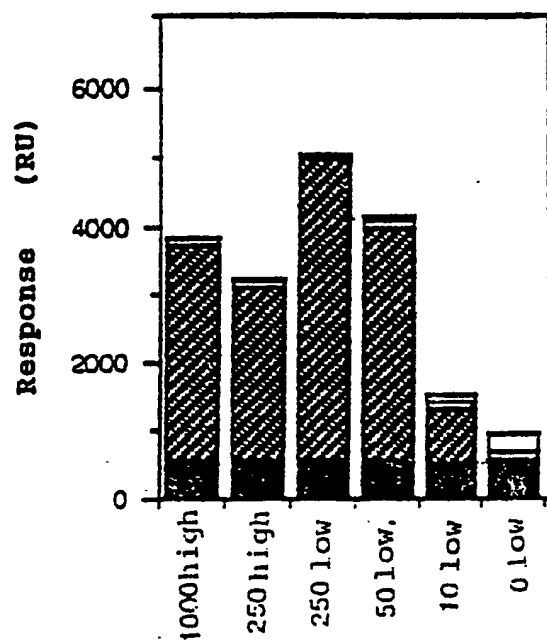
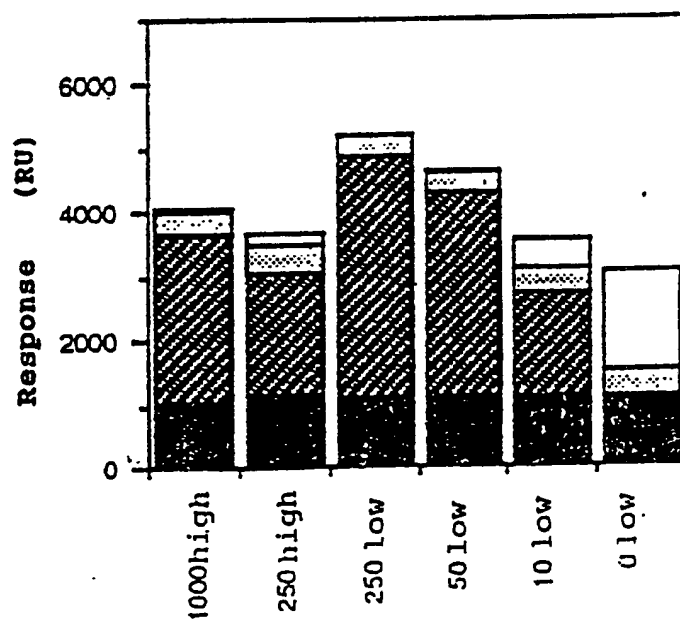


FIG. 6



Block conc. (µg/ml)/ion strength

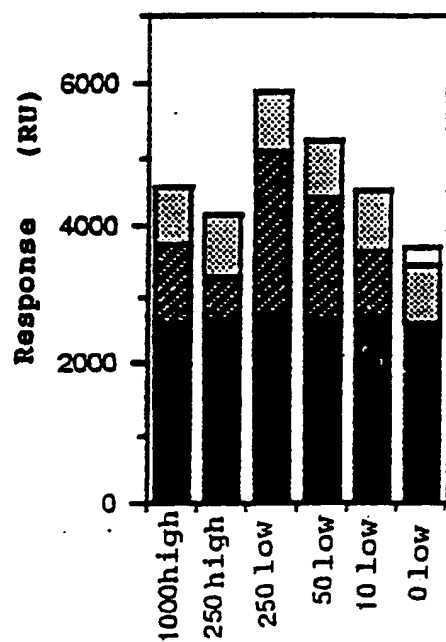
FIG. 7



Block conc. (µg/ml)/ion strength

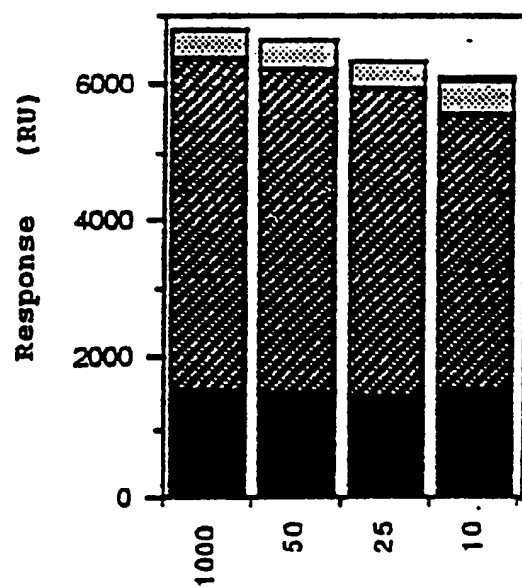
FIG. 8

5/5



Block conc. (μg/ml)/ion strength

FIG.9



Block conc. (μg/ml)

FIG.10

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 91/00649

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: G 01 N 33/545, 33/53														
II. FIELDS SEARCHED <div style="text-align: center; border: 1px solid black; padding: 2px;">Minimum Documentation Searched⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border: 1px solid black; padding: 2px;">Classification System</th> <th style="border: 1px solid black; padding: 2px;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; padding: 5px; vertical-align: top;">IPC5</td> <td style="border: 1px solid black; padding: 5px; vertical-align: top;">G 01 N; C 07 K</td> </tr> </table> <div style="text-align: center; border: 1px solid black; padding: 2px;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in Fields Searched⁸</div>			Classification System	Classification Symbols	IPC5	G 01 N; C 07 K								
Classification System	Classification Symbols													
IPC5	G 01 N; C 07 K													
SE,DK,FI,NO classes as above														
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 2px;">Category *</th> <th style="width: 60%; padding: 2px;">Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹²</th> <th style="width: 30%; padding: 2px;">Relevant to Claim No.¹³</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;"> WO, A1, 8302954 (VENTREX LABORATORIES, INC.) 1 September 1983, see in particular claims 1-5, 19-27; abstract <div style="text-align: center;">--</div> </td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-10</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;"> WO, A1, 9005303 (PHARMACIA AB) 17 May 1990, see the whole document <div style="text-align: center;">--</div> </td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-10</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;"> Dialog Information Service, file 155: MEDLINE 66-91 April, Accession no. 88285764, HC Graves et al: "The effect of surface charge on non-specific binding of rabbit immunoglobulin G in solid-phase immunoassays", & J Immunol Methods Jul 22 1988, 111 (2) p 157-66 <div style="text-align: center;">--</div> </td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-10</td> </tr> </tbody> </table>			Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	A	WO, A1, 8302954 (VENTREX LABORATORIES, INC.) 1 September 1983, see in particular claims 1-5, 19-27; abstract <div style="text-align: center;">--</div>	1-10	A	WO, A1, 9005303 (PHARMACIA AB) 17 May 1990, see the whole document <div style="text-align: center;">--</div>	1-10	A	Dialog Information Service, file 155: MEDLINE 66-91 April, Accession no. 88285764, HC Graves et al: "The effect of surface charge on non-specific binding of rabbit immunoglobulin G in solid-phase immunoassays", & J Immunol Methods Jul 22 1988, 111 (2) p 157-66 <div style="text-align: center;">--</div>	1-10
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³												
A	WO, A1, 8302954 (VENTREX LABORATORIES, INC.) 1 September 1983, see in particular claims 1-5, 19-27; abstract <div style="text-align: center;">--</div>	1-10												
A	WO, A1, 9005303 (PHARMACIA AB) 17 May 1990, see the whole document <div style="text-align: center;">--</div>	1-10												
A	Dialog Information Service, file 155: MEDLINE 66-91 April, Accession no. 88285764, HC Graves et al: "The effect of surface charge on non-specific binding of rabbit immunoglobulin G in solid-phase immunoassays", & J Immunol Methods Jul 22 1988, 111 (2) p 157-66 <div style="text-align: center;">--</div>	1-10												
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁰</p> <p>^{"A"} document defining the general state of the art which is not considered to be of particular relevance</p> <p>^{"E"} earlier document but published on or after the international filing date</p> <p>^{"L"} document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>^{"O"} document referring to an oral disclosure, use, exhibition or other means</p> <p>^{"P"} document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>^{"T"} later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>^{"X"} document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>^{"Y"} document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>^{"&"} document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search 13th December 1991 </td> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report 1992 -01- 13 </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;"> International Searching Authority <div style="text-align: center; margin-top: 10px;">SWEDISH PATENT OFFICE</div> </td> <td style="border: 1px solid black; padding: 5px;"> Signature of Authorized Officer <div style="text-align: center; margin-top: 10px;"> Kerstin Boije Jansson </div> </td> </tr> </table>			Date of the Actual Completion of the International Search 13th December 1991	Date of Mailing of this International Search Report 1992 -01- 13	International Searching Authority <div style="text-align: center; margin-top: 10px;">SWEDISH PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center; margin-top: 10px;"> Kerstin Boije Jansson </div>								
Date of the Actual Completion of the International Search 13th December 1991	Date of Mailing of this International Search Report 1992 -01- 13													
International Searching Authority <div style="text-align: center; margin-top: 10px;">SWEDISH PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center; margin-top: 10px;"> Kerstin Boije Jansson </div>													

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	US, A, 4829009 (HOWARD GRAVES) 9 May 1989, see column 5, line 54; column 7, line 69; claims --	1-10
P,A	EP, A1, 0406473 (ABBOTT LABORATORIES) 9 January 1991, see the whole document -- -----	1-10

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/SE 91/00649**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 31/10/91. The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 8302954	83-09-01	EP-A- 0101724	84-03-07
WO-A1- 9005303	90-05-17	SE-B-C- 462454 SE-A- 8804073	90-06-25 88-11-10
US-A- 4829009	89-05-09	NONE	
EP-A1- 0406473	91-01-09	AU-D- 3891389 JP-A- 3044399	89-11-02 91-02-26